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Receptors for Sensory Neuropeptides in Human Inflammatory Diseases: Implications for the Effector Role of Sensory Neurons[1]

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MANTYH, P. W., M. D. CATTON, C. G. BOEHMER, M. L. WELTON, E. P. PASSARO, JR., J. E. MAGGIO AND S. R. VIGNA. *Receptors for sensory neuropeptides in human inflammatory diseases: Implications for the effector role of sensory neurons.* PEPTIDES **10**(3) 627–645, 1989.—Glutamate and several neuropeptides are synthesized and released by subpopulations of primary afferent neurons. These sensory neurons play a role in regulating the inflammatory and immune responses in peripheral tissues. Using quantitative receptor autoradiography we have explored what changes occur in the location and concentration of receptor binding sites for sensory neurotransmitters in the colon in two human inflammatory diseases, ulcerative colitis and Crohn's disease. The sensory neurotransmitter receptors examined included bombesin, calcitonin gene related peptide-α, cholecystokinin, galanin, glutamate, somatostatin, neurokinin A (substance K), substance P, and vasoactive intestinal polypeptide. Of the nine receptor binding sites examined only substance P binding sites associated with arterioles, venules and lymph nodules were dramatically up-regulated in the inflamed tissue. These data suggest that substance P is involved in regulating the inflammatory and immune responses in human inflammatory diseases and indicate a specificity of efferent action for each sensory neurotransmitter in peripheral tissues.

Inflammatory bowel disease Receptors Sensory neuropeptides Substance P Vasoactive intestinal peptide

NEURONS that convey sensory information from peripheral tissues to the central nervous system are located in the dorsal root ganglion (DRG); as such they are known as DRG neurons. Recently, several neuropeptides including calcitonin gene related peptide-α and -β, cholecystokinin, galanin, gastrin releasing peptide (the mammalian analogue of bombesin), somatostatin, neurokinin A (also known as substance K), substance P (SP), and vasoactive intestinal peptide have each been shown to be present in various subpopulations of DRG neurons (28,62). Several of these neuropeptides have been implicated in the conduction of nociceptive information. For example: intrathecal injection of SP, the best studied of these peptides, produces biting and scratching behavior consistent with a role for SP as a peptide neurotransmitter of some primary afferent nociceptors (24); SP release in the spinal cord is inhibited by opiate analgesics (26); depletion of SP by capsaicin [a neurotoxin relatively selective for unmyelinated sensory neurons (63) including those containing SP] roughly parallels a loss of specific nociceptive response; and release of SP in the spinal dorsal horn in response to normally innocuous mechanical stimuli is enhanced in polyarthritic rats (65).

It has become increasingly evident in the last decade that the DRG neurons which convey *afferent* somatosensory information from peripheral tissues to the spinal cord are also involved in the *efferent* regulation of the peripheral tissues they innervate. Thus, SP-containing DRG neurons have been implicated in both the *afferent* central transmission of nociceptive information and in the *efferent* regulation of inflammation and sensitization of joint sensory endings in a chronic pain state such as arthritis (31–33).

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ABBREVIATIONS

A	artery	Lym	lymph nodule
bas	basal	Mc	mucosa
BBS	bombesin	Mc-b	mucosa, basal aspect
bv	blood vessel (arteriole or venule)	Mc-l	mucosa, luminal aspect
CCK	cholecystokinin	MM	muscularis mucosa
CGRP	calcitonin gene related peptide-α	MP	myenteric plexus
CM	circular muscle of the muscularis externa	musc	muscle
ct	connective tissue	p	proliferative zone of a lymph nodule
g	germinal layer of lymph nodule	SK	substance K, neurokinin A, α-neurokinin, neuromedin L
GAL	galanin	SOM	somatostatin
GLU	glutamate	SP	substance P
H-E	hematoxylin and eosin	SubMc	submucosa
LM	longitudinal muscle of the muscularis externa	U.C.	ulcerative colitis
lum	luminal	VIP	vasoactive intestinal peptide

Support for this concept includes observations that: a) SP is a potent vasodilator (32), b) terminals of SP-containing sensory neurons are observed in close apposition to blood vessels (37), and c) electrical stimulation of peripheral nerves at intensities that release SP reproduces many of the physiological changes seen in acute inflammation (19). In rats with experimentally-induced arthritis, SP concentrations increase in peripheral nerves innervating the affected joints (31), and if capsaicin is administered to rats before or after the onset of arthritis, the attendant paw swelling and tenderness is diminished (9).

The hypothesis that sensory neurons containing SP and/or other neuropeptides are involved in regulating the inflammatory and immune responses in the peripheral tissues they innervate suggests that, in a pathological state, these neuropeptide-containing sensory neurons may contribute to an altered or abnormal inflammatory response such as that seen in arthritis or inflammatory bowel diseases. In the present report we have used quantitative receptor autoradiography to determine whether receptor binding sites for putative sensory neurotransmitters (neuropeptides and the excitatory amino acid glutamate) are altered in a human inflammatory disease.

The human inflammatory disease we have explored is inflammatory bowel disease (IBD), which is a generic term that refers to chronic inflammatory diseases of the intestine which are of unknown etiology, principally ulcerative colitis and Crohn's disease (70). Ulcerative colitis is an inflammatory, ulcerating process of the colon; Crohn's disease is an inflammation of the intestine characterized by nodular granulomatous inflammatory lesions throughout the entire gut wall that may involve any part of the intestine but primarily attacks the distal small intestine and colon. We chose to explore alterations in receptors for sensory neurotransmitters in these diseases since it has previously been demonstrated that the gastrointestinal tract is innervated by sensory fibers that contain and release neuropeptides including SP (3, 16, 71, 73, 78, 79). In addition, IBD is probably one of the few human inflammatory diseases where surgical removal of inflamed intestinal tissue is used to ameliorate the symptoms in severe cases, thus making quantitative receptor autoradiographic analysis possible. The rationale for the experiments is that if a particular neurotransmitter is involved in the peripheral inflammatory response, then some alteration in the expression of its receptors may be anticipated. The present report is an exploration of sensory neurotransmitter receptor binding sites in inflamed and normal human gastrointestinal tissue in order to determine whether changes in the location and/or apparent density of these sites are correlated with these diseases.

EXPERIMENTAL PROCEDURES

Human Specimens

Specimens were obtained within 5 minutes after removal,

TABLE 1

DETAILS OF PATIENTS

Group	No.	Mean Age (year)	Sex M	Sex F	Patient's Weight (kg)	Specimen Site: Ileum	Specimen Site: Ileum and Colon	Specimen Site: Colon	Patients With No Steroid Treatment
IBD	8	35(15–58)*	2	6	63(47–82)†	1	3	5	2
Crohn's	4	31(15–44)	1	3	52(47–59)	1	3	1	2
U.C.	4	35(20–58)	1	3	66(54–82)	—	—	4	0
Carcinoma resection	6	68(62–78)	6	0	76(66–82)	—	2	4	6

*Numbers in parentheses are ranges of patient's age. †Numbers in parentheses are ranges of patient's weights. Results from the ileum were the same as the colon (i.e., arterioles, venules and lymph nodules in inflamed tissue contain high concentrations of substance P receptor binding sites) and there was no statistically significant difference between results from IBD steroid-treated and untreated patients.

embedded in Tissue-Tek (Miles) and placed in dry ice to minimize postsurgical degradation artifacts. The specimens (Table 1) obtained at the margins of resection for carcinoma (n = 6) were obtained as far from the tumor as possible; specimens obtained from Crohn's disease (n = 4) and ulcerative colitis (n = 4) patients were from areas with the highest degree of inflammation. In all cases the diagnosis was independently determined by a pathologist to be either ulcerative colitis, Crohn's disease, or histologically normal at the site of resection. The tissue was then blocked, placed on a brass microtome chuck, frozen on dry ice and processed for quantitative autoradiography as previously described (48, 51, 52). The tissue was serially sectioned (30 μm), thaw-mounted onto gelatin-coated microscope slides and stored at −70°C over desiccant for no longer than 3 months.

Radioligands

The radioligands used in the present study were ^{125}I-labeled synthetic peptides purified by reverse-phase HPLC to essentially quantitative specific activity (approximately 2000 Ci/mmol).

Receptor Binding Protocols

Quantitative receptor autoradiography was performed by first bringing the slide-mounted tissue sections to room temperature. The slide-mounted tissue sections were then placed consecutively in a preincubation medium, an incubation medium, a wash solution, and a final dip in distilled water. The preincubations and washes were performed by immersing the entire slide in the appropriate solution whereas the incubation with the radioligand was performed by placing the slides on a flat surface and covering the sections with 1.5 ml of the incubation medium. To determine the nonspecific binding, paired serial sections were incubated as described above except that a 1 μM concentration of the appropriate nonradioactive peptide was added to the incubation solution. Each ligand required a unique set of binding conditions which are given below.

Bombesin

Sections were first preincubated in 10 mM HEPES (pH 7.4) for 5 min followed by an incubation in a solution of 10 mM HEPES (pH 7.4), 4.7 mM KCl, 130 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.1% BSA, 100 mg/ml bacitracin and 100 pM of ^{125}I-[Tyr^4]-bombesin for 1 hour. After the incubation the sections were washed (4 times, 2 min each) in a solution of 10 mM HEPES (pH 7.4) and 0.01% BSA (80).

Calcitonin Gene Related Peptide-α

Sections were preincubated in 50 mM Tris-HCl buffer (pH 7.4) for 5 minutes followed by an incubation in a solution of 50 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, 2 mM EGTA and 100 pM of ^{125}I-iodohistidyl^{10}-human-CGRP-α (Amersham) for 2 hr. After this the sections were washed (4 times, 3 min each) in a solution of 50 mM Tris-HCl (pH 7.4), 0.1% BSA (53).

Cholecystokinin

Sections were preincubated for 10 min in 50 mM Tris-HCl buffer (pH 7.7, 20°C) followed by an incubation in the same buffer with 5 mM $MgCl_2$, 0.2% BSA, 0.02% bacitracin, 1 mM dithiothreitol and 100 pM ^{125}I-Bolton-Hunter CCK-8 sulfated (Amersham) for 60 min. After this the sections were washed (4 times, 3 min each) in the same buffer containing 0.1% BSA (46).

Galanin

Sections were preincubated for 10 min in 10 mM HEPES buffer (pH 7.4, 20°C) followed by an incubation in the same HEPES buffer with 100 pM ^{125}I-galanin (monoiodinated porcine galanin labeled using chloramine-T) added for 1 hr. The sections were then washed (4 times, 3 min each) in the same buffer (60).

Glutamate

Sections were incubated for 45 min at 4°C in 200 nM L-[^{3}H]glutamate (diluted with unlabeled glutamate to a "working" specific activity of 4.5 Ci/mmol, in order to label both the high and low affinity receptor binding sites) in 50 mM Tris-HCl (pH 7.4) containing 2.5 mM $CaCl_2$. Nonspecific binding was determined in the presence of 1 mM unlabeled glutamate. After the incubation, sections were rinsed three times with cold buffer, then rinsed twice with 2 ml of cold 2.5% glutaraldehyde in neat acetone, to minimize dissociation during drying. The total rinse time was approximately 10 sec (20).

Somatostatin

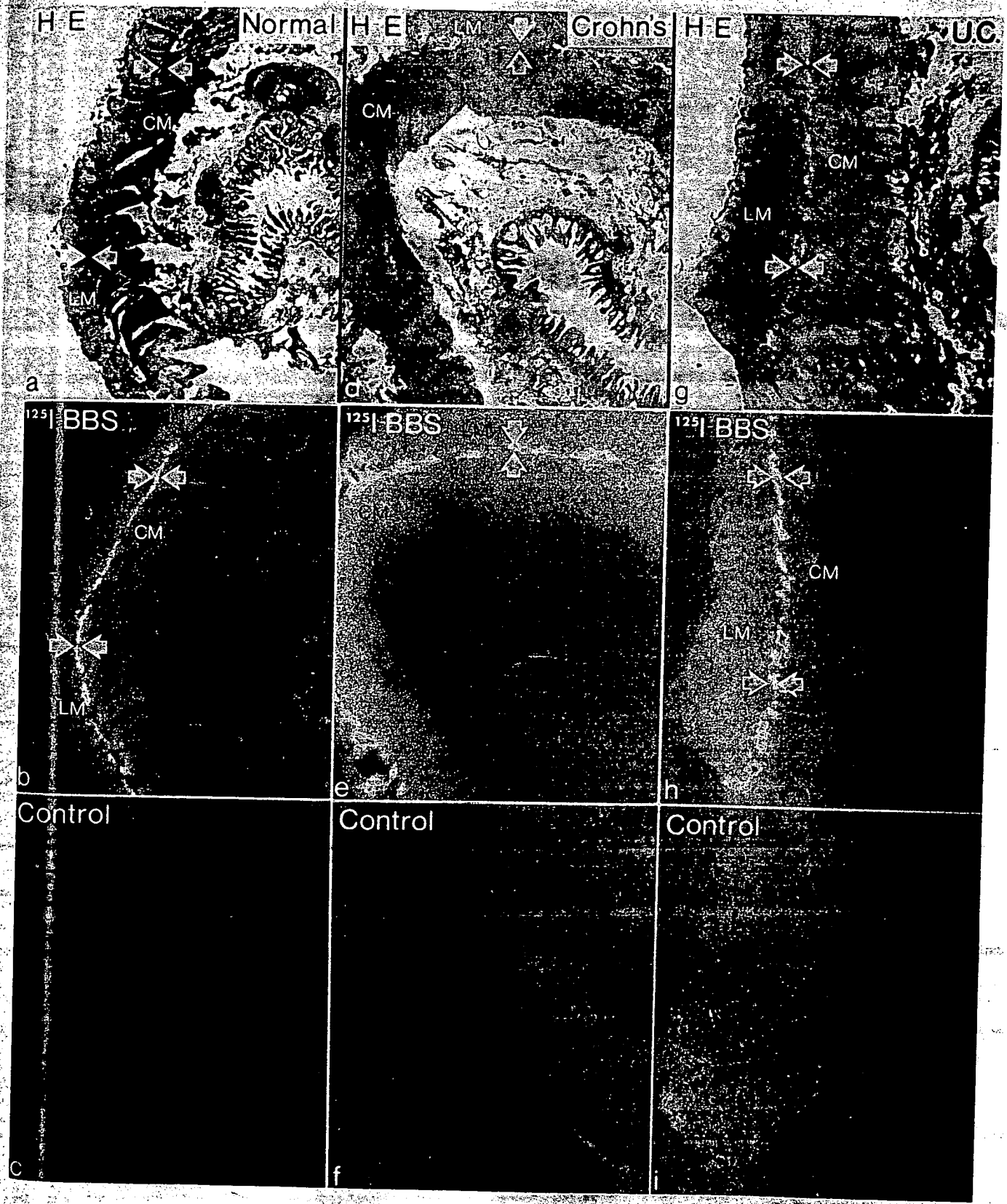
Sections were preincubated in 70 mM Tris-HCl for 5 min and then in an incubation solution of 170 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1% BSA, 20 mg/l bacitracin, and 100 pM ^{125}I-[Tyr^3]-somatostatin-8 cyclic analog (D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Cys-Thr-NH_2) [which is similar to Sandostatin® (59)] for 2 hr. The sections were then washed in 170 mM Tris-HCl buffer (pH 7.4) and 0.01% BSA (4 times, 4 min each).

Neurokinin A (Substance K)

Sections were brought to room temperature and placed in a preincubation medium (19°C for 10 min) consisting of 50 mM Tris-HCl, pH 7.4. They were then incubated at 10°C for 2 hr in a

FOLLOWING PAGE

FIG. 1. A series of lightfield and darkfield photomicrographs showing localization of bombesin (BBS) receptor binding sites in transverse sections of the human colon from normal (a, b, c), Crohn's disease (d, e, f) and ulcerative colitis (g, h, i) patients. In these and the following plates (Figs. 1, 3, 5, 6 and 10), a, d and g are brightfield photomicrographs of sections stained with hematoxylin and eosin (H&E stain), b, e and h are autoradiograms which show the total binding and c, f and i show the nonspecific binding. Control sections (nonspecific binding) were treated identically to the sections which show the total binding (b, e and h) except that 1 μM of the appropriate unlabeled peptide (in this case BBS) was added to the incubation medium. In all the darkfield autoradiograms the white silver grains represent concentrations of binding sites. To obtain the *specific* binding for the normal colon the binding in (c) was subtracted from that in (b), for the Crohn's disease colon (f) was subtracted from (e), and for the ulcerative colitis (U.C.) colon (i) was subtracted from (h). Note that in surgical specimens of the normal, Crohn's disease, and U.C. colon a moderate concentration of specific BBS binding sites is present over the neruons of the myenteric plexus (indicated by arrows) whereas a low concentration is present over the external circular and longitudinal muscle. Also note that there does not appear to be a significant difference in either the location or density of specific sites between tissues from the normal, Crohn's disease and U.C. patients. Line bar = 0.7 mm.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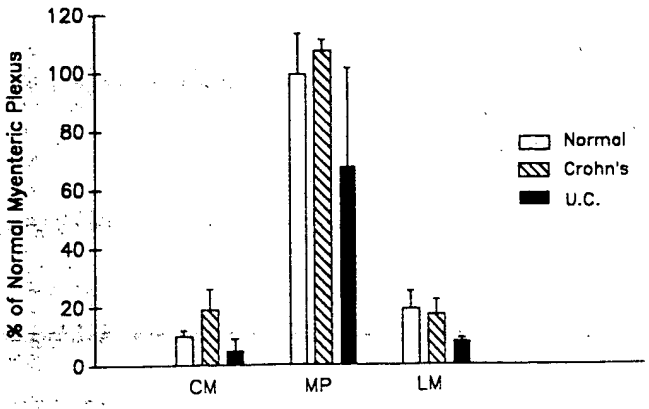

FIG. 2. Histogram showing relative concentration of bombesin binding sites comparing the normal (open bar), Crohn's disease (hatched bar) and ulcerative colitis (U.C., filled bar) tissue. In this and the following histograms (Figs. 4, 7 and 11) the tissue with the highest density of binding sites for each radioligand in the normal colon is assigned a value of 100% and all other tissues which express simlar binding sites are expressed as a percentage of this value. In the case of bombesin in the normal tissue the highest concentration of specific binding sites is the myenteric plexus. Note that there are no significant changes in the density of binding sites for bombesin (when comparing the normal, Crohn's or U.C. tissue) in the external circular muscle (CM), myenteric plexus (MP), or the external longitudinal muscle (LM).

solution of 50 mM Tris-HCl, pH 8.0, containing 3 mM $MnCl_2$, 200 mg/l bovine serum albumin, 2 mg/l chymostatin, 4 mg/l leupeptin, 40 mg/l bacitracin, and 100 pM of ^{125}I-Bolton-Hunter neurokinin A (substance K). Following this incubation, the sections were rinsed with four washes of 50 mM Tris-HCl, pH 7.4 (4°C, 5 min each) and two washes of distilled water (4°C, 5 sec each) (47, 52, 55).

Substance P

Sections were brought to room temperature and placed in a preincubation medium (20°C for 10 min) consisting of 50 mM Tris-HCl, pH 7.4, containing 0.005% (v/v) polyethylenimine. The sections were then incubated at 19°C for 1 hr in a solution of 50 mM Tris-HCl, pH 7.4, containing 3 mM $MnCl_2$, 200 mg/l bovine serum albumin, 2 mg/l chymostatin, 4 mg/l leupeptin, 40 mg/l bacitracin, and 100 pM of ^{125}I-Bolton-Hunter substance P. Following this incubation, the sections were rinsed with four washes of 50 mM Tris-HCl, pH 7.4 (4°C, 2 min each) and two washes of distilled water (4°C, 5 sec each) (48, 49, 51, 54).

Vasoactive Intestinal Polypeptide

Sections were preincubated in 10 mM HEPES buffer (pH 7.4) for 5 minutes at 20°C followed by incubation in a solution of 10 mM Tris-HCl buffer (pH 7.4), 130 mM NaCl, 4.7 mM KCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM EGTA, 1% BSA, 1 mg/ml bacitracin and 100 pM ^{125}I-iodotyrosyl^{10}-VIP (Amersham) for 2 hr at 20°C. After this the sections were washed (twice for 15 min, 20°C) in the incubation solution minus the radioligand.

Analysis of Autoradiograms

After the final wash all the sections were dipped in distilled water, dried in the cold room (4°C), and stored overnight in desiccant. Quantitative autoradiographic analysis was performed by placing the dried, labeled sections in apposition to tritium-sensitive film (Ultrofilm, LKB or Hyperfilm, Amersham) along with iodinated brain mash or commerically available standards (Amersham). After 1–4 weeks; the film was developed in Kodak D-19 developer, fixed, and washed. In sections where a higher degree of histological resolution of the binding sites was sought, the tissues slices were overlaid with emulsion-coated coverslips or processed for standard emulsion-dipped autoradiography. After these autoradiograms were developed, the sections were placed in Carnoy's fixative for 3 hr, stained with hemotoxylin and eosin (H&E) and mounted with Histoclad. Darkfield or brightfield photomicrographs were then taken of the silver grains and counterstained sections, respectively. Using this approach three complementary images were generated: the autoradiograms which were analyzed by quantitative densitometry, the autoradiograms of the emulsion-dipped slides which provided detailed histological resolution of the binding sites, and the counterstained section which allowed identification of the cell type expressing a specific binding site. Controls for chemographic artifacts were generated by performing the binding exactly as described except that the radioligand was omitted from the incubation medium.

Analysis of Data

To quantitate the density of radiolabeled neurotransmitter binding sites, microdensitometry with tritium-sensitive film was performed. The exposed film was projected at 20× on a white horizontal surface and the density of the projected image measured with a photocell connected to a digital voltmeter as described (54). At 20× the resolution of the device corresponds to a region 20 μm in diameter on the projected sections. Previous experiments have established that the LKB film does not respond linearly to a linear increase in radioactivity. We therefore constructed a series of standards, exposed these to LKB film, developed and fixed the film, measured this film densitometrically and used these values with a Texas Instruments automatic curve fitting program to obtain a description of film characteristics and correct for the nonlinearity. In all cases, specific binding was obtained by subtracting nonspecific binding from the total binding. Nonspecific binding was defined as that binding remaining in the presence of a 1 μM concentration of the unlabeled peptide.

Statistical Analysis

The results were expressed as mean ± one standard error of the mean (SEM) and examined for statistical signficance using the Student's *t*-test for independent samples. In all histograms (Figs. 2, 4, 9, and 11) only differences with a significance greater than $p \leq 0.05$ are indicated.

RESULTS

Bombesin

In the normal human colon, receptor binding sites for bombesin (^{125}I-[Tyr^4]-bombesin) are present in high amounts over neurons of the myenteric plexus and in low amounts over both the circular muscle and longitudinal muscle of the muscularis externa (Fig. 1). In comparing histologically normal tissue with tissue obtained from patients with either Crohn's disease or ulcerative colitis, no significant changes were observed in the density of receptor binding sites in the myenteric plexus, circular, or longitudinal muscle (Fig. 2 and Table 2).

Calcitonin Gene Related Peptide-α

In both the histologically normal tissue and the tissues from

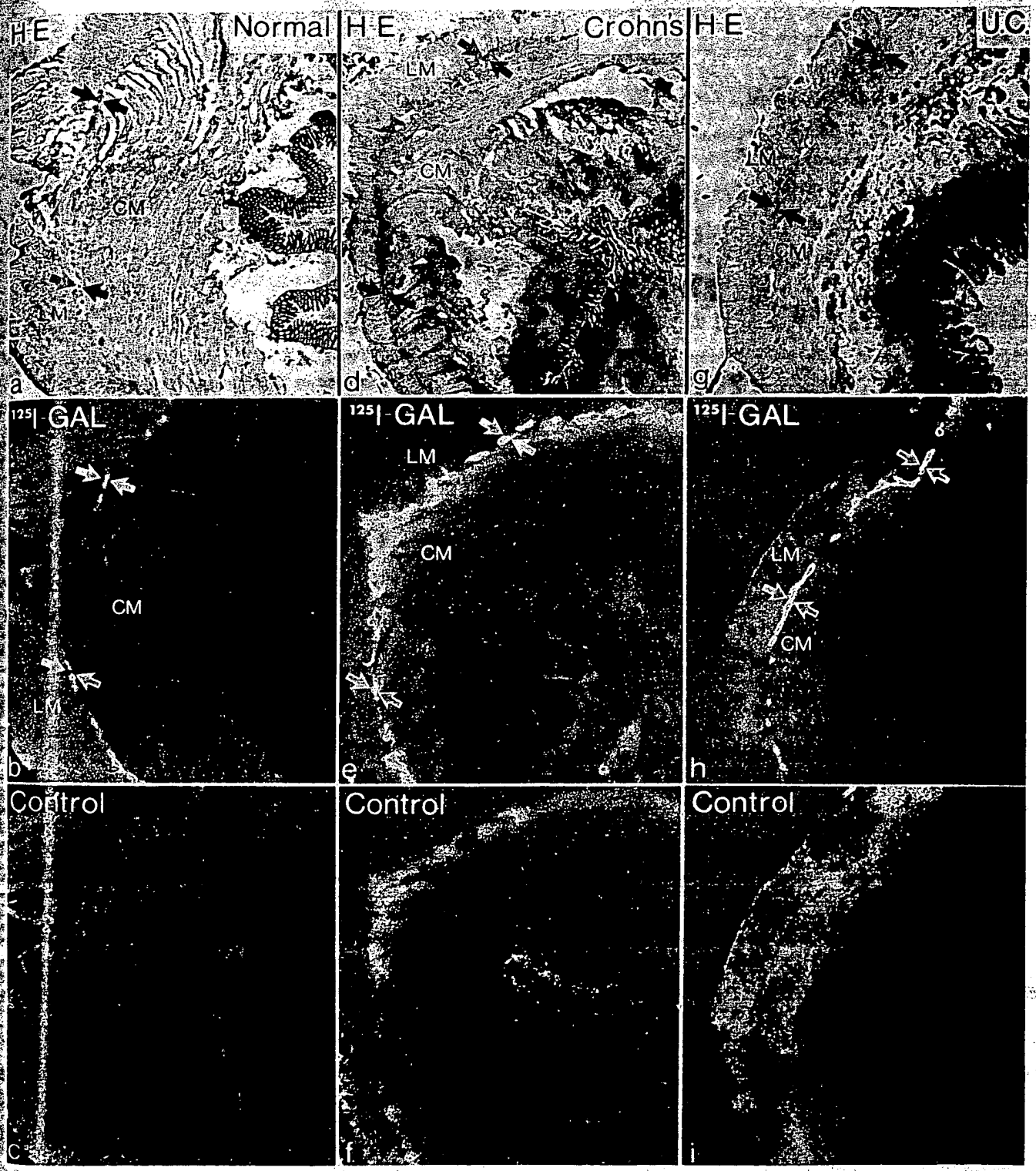

FIG. 3. A series of light and darkfield photomicrographs showing localization of galanin (GAL) receptor binding sites in transverse sections of the human colon from normal (a, b, c), Crohn's disease (d, e, f) and ulcerative colitis (g, h, i) patients. The only specific binding sites are over the myenteric plexus. Note also that the density of binding sites does not appear to differ significantly between the tissue from the normal, Crohn's disease, and ulcerative colitis patients. See Fig. 1 for further explanation. Line bar = 0.7 mm.

TABLE 2

RELATIVE CONCENTRATION OF BINDING SITES FOR ALL RADIOLIGANDS TESTED COMPARING THE NORMAL (N), CROHN'S (CR) AND ULCERATIVE (UC) TISSUE

	BBS				CGRP			GAL				NKA (SK)				SP			VIP		
	N	Cr	UC	CCK	N	Cr	UC	N	Cr	UC	GLU	N	Cr	UC	SOM	N	Cr	UC	N	Cr	UC
Mc																					
lum	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.0	1.0	*0.1*
bas	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.3	0.2	*0.1*
SubMc																					
bv	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1	*0.7*	*0.9*	—	—	—
ct	—	—	—	—	1.0	0.8	1.0	—	—	—	—	—	—	—	—	—	—	—	—	—	—
lym	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.0	*1.1*	*1.1*	—	—	—
CM																					
bv	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.0	*0.9*	*1.2*	—	—	—
musc	0.1	0.2	0.1	—	—	—	—	—	—	—	—	1.0	2.0	1.6	—	1.0	0.8	0.6	0.2	0.1	0.2
MP	1.0	1.1	0.7	—	—	—	—	1.0	1.3	1.2	—	—	—	—	—	—	—	—	0.3	0.1	0.3
LM																					
bv	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.0	*1.0*	*1.2*	—	—	—
musc	0.2	0.2	0.1	—	—	—	—	—	—	—	—	0.3	0.8	0.6	—	—	—	—	0.2	0.2	0.2

In the normal colon the tissue with the higest density of binding sites for each radioligand is assigned a value of 1.0 and all other tissues which contain similar binding sites are expressed as a ratio of this value. Tissues which show a significant change in the density of binding sites compared to normal are italic.

Crohn's and ulcerative colitis patients, low levels of specific receptor binding sites for CGRP (^{125}I-iodohystidyl^{10}-human CGRP-α) were present over the stroma of the submucosa, which is primarily composed of connective tissue. There was no difference in the density of specific binding sites in comparing the histologically normal tissue and the tissue from Crohn's or ulcerative colitis patients (Fig. 4 and Table 2). It should be noted that while there is a differential expression of CGRP-α and CGRP-β by primary sensory neurons and enteric autonomic neurons in the rat, these two peptides appear to interact with the same receptor binding site in the rat colon (62).

Cholecystokinin

Specific binding sites for cholecystokinin (^{125}I-Bolton-Hunter

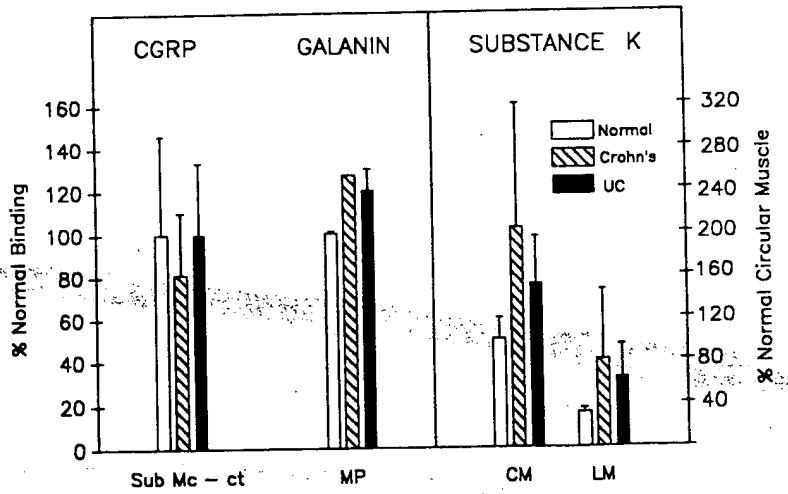

FIG. 4. Histogram showing the changes in the location and concentration of specific binding sites for calcitonin gene related peptide α (CGRP), galanin (GAL), and neurokinin A (also known as substance K (SK). Specific CGRP binding sites were located over the connective tissue in the submucosa (SubMc-ct), specfic binding sites for galanin (GAL) were located over the myenteric plexus (MP), and specific binding sites for neurokinin A (substance K) were located over the external circular (CM) and longitudinal muscle (LM). Note that while each ligand binding site is present over a different set of cell types, there were no significant differences in the densities of CGRP, GAL or SK binding sites between the tissues obtained from the normal, Crohn's disease,or ulcerative colitis patients. See Fig. 2 for further explanation.

CCK-8 sulfated) were not observed in any area of the human colon (Table 2), although specific binding sites for the same radioligand were observed in canine fundus and the rat brain under identical experimental conditions (Mantyh, unpublished observations).

Galanin

Specific binding sites for galanin (^{125}I-porcine galanin) were located over the myenteric plexus in the normal, Crohn's disease, and ulcerative colitis samples (Fig. 3). No significant difference in the density of binding sites was noted among these tissues (Fig. 4 and Table 2).

Glutamate

Specific binding sites for glutamate (^{3}H-glutamate) were not observed in any area of the human colon (Table 2) although specific binding sites using the same radioligand and experimental conditions were observed in the spinal cord and brainstem of the rat, rabbit, cat and monkey (Mantyh, unpublished observations).

Neurokinin A (Substance K)

Specific neurokinin A (^{125}I-Bolton-Hunter neurokinin A) receptor binding sites are present in low levels over the external circular and longitudinal muscles from the histologically normal tissue and tissue obtained from Crohn's disease or ulcerative colitis patients (Fig. 5). No change in the density of these receptors was evident in comparing the normal and inflamed colon (Fig. 4 and Table 2).

Somatostatin

Specific somatostatin binding sites (using a cyclic analog of ^{125}I-somatostatin-8) were not observed in any areas of the human colon (Table 2) although specific binding sites for the same radioligand were observed in the brain, spinal cord, and brainstem of the rat, rabbit, cat, and monkey using identical experimental conditions (Mantyh, unpublished observations). It should be noted, however, that this analog, which is not rapidly degraded and is therefore stable enough to be used with receptor autoradiography, is known to bind only to a subpopulation of somatostatin receptors (59).

Substance P

In the histologically normal colon; moderate levels of SP (^{125}I-Bolton-Hunter substance P) receptor binding sites are present over the external circular muscle and over smooth muscle cells comprising the tunica media of the large arteries just outside the serosa (Fig. 6). In addition, a low concentration of SP binding sites is found on arterioles and venules in the submucosa.

In tissues obtained from patients with Crohn's disease there was a striking change in the distribution and levels of SP receptor binding sites compared to that seen in the histologically normal surgical specimens obtained at the margins of extensive resections for carcinoma (Fig. 6). The most notable difference is seen in SP receptor binding sites present over arterioles (Figs. 6 and 7), venules, and lymph nodules (Figs. 6 and 8). In tissues from patients with IBD, arterioles and venules (diameter 0.1–1.0 mm) in all layers of the colon express very high levels of SP receptor binding sites whereas in normal colon tissue, SP receptor binding sites are undetectable in blood vessels (with the exception of the occasional large arteries just inside the serosa or the arterioles and venules in the submucosa, the latter two of which express low levels of SP binding sites). Since it is difficult to identify the total number of arterioles and venules in H&E-stained sections, we have instead compared the total number of blood vessels expressing SP binding sites in the histologically normal vs. disease specimens in a defined comparable area (2.6 mm by 3.5 mm) of the tissue sections. Comparing the average number of arterioles and venules exhibiting SP receptor binding sites, SP receptor binding sites in samples from 6 normal vs. 4 Crohn's disease patients revealed 6.5 ± 2.4 vs. 14.5 ± 4.9 in the submucosa, 0 vs. 14 ± 1.4 ($p<0.01$) in the external circular muscle and 0 vs. 18.5 ± 3.5 ($p<0.01$) in the external longitudinal muscle, respectively.

Coincident with the ectopic expression of SP receptor binding sites by arterioles (Fig. 7) and venules in inflamed tissue is the expression of SP receptor binding sites by the lymph nodules which border the muscularis mucosa. Within each lymph nodule only cells associated with the lightly stained (by H&E) germinal center display detectable SP receptor binding sites (Fig. 8). In 36 lymph nodules found in surgical specimens obtained from patients with Crohn's disease, all 36 display high concentrations of SP binding sites. In contrast, SP binding sites were undetectable in the 24 lymph nodules localized in histologically normal surgical specimens from the 6 patients with carcinoma resection. In IBD tissues, the external circular muscle displays a slightly lower level of SP binding sites than in normal tissues (Figs. 6 and 9), but this decrease is not statistically significant.

Surgical material from ulcerative colitis patients revealed a pattern and level of SP receptor binding sites similar to that observed in the specimens obtained from Crohn's disease patients (Figs. 6–9). In specimens from ulcerative colitis patients, very high levels of SP receptor binding sites are present over both small arterioles (Fig. 7) and venules located in the muscularis mucosa, submucosa, external circular muscle, external longitudinal muscle, and the serosa. In samples of normal tissue obtained from 6 carcinoma resection patients vs. inflamed tissue from 4 patients with ulcerative colitis, the average number of arterioles and venules in the histologically normal vs. ulcerative colitis tissue in a 2.6 mm by 3.5 mm sample was 6.5 ± 2.3 vs. 13.3 ± 1.5 in the submucosa, 0 vs. 15 ± 6.2 ($p<0.01$) in the external circular muscle and 0 vs. 19 ± 5 ($p<0.01$) in the external longitudinal muscle, respectively.

The germinal centers of lymph nodules in tissue obtained from ulcerative colitis patients also displayed very high levels of SP receptor binding sites compared to those found in normal tissue (Fig. 6–9). Thus, of the 29 lymph nodes in H&E-stained sections of the 4 cases of ulcerative colitis, 28 expressed very high levels of SP binding sites, whereas 24 nolules localized in normal colon failed to express detectable levels of SP binding sites.

Vasoactive Intestinal Polypeptide

In the normal human colon a high level of VIP receptor binding

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FIG. 5. A series of light and darkfield photomicrographs showing the localization of receptor binding sites for neurokinin A [also known as substance K (SK)] in transverse sections of the human colon from normal (a, b, c), Crohn's disease (d, e, f) and ulcerative colitis (g, h, i) patients. The only specific binding sites are over the external circular and longitudinal muscle. Note also that the density of binding sites does not appear to differ significantly between the tissue obtained from the normal, Crohn's disease and ulcerative colitis patients. See Fig. 1 for further explanation. Line bar = 0.7 mm.


H-E
Normal
SubMc
MM
CM
LM
H-E
Crohn's
CM
SubMc
LM
H-E
U.C.
SubM
LM
125I-SK
SK
SubMc
MM
CM
LM
b
125I-SK
CM
SubMc
LM
e
125I-SK
SubMc
CM
LM
h
Control
c
Control
f
Control
i

sites is present over the luminal portion of the mucosa whereas a low density of receptor binding sites is present over the basal portion of the mucosa, the myenteric plexus, and the circular and longitudinal muscles (Figs. 10 and 11). A comparison of histologically normal tissue with tissue obtained from Crohn's disease patients revealed no significant difference in the density of binding sites in any area of the colon. However, when the histologically normal tissue was compared to tissue obtained from ulcerative colitis patients (Figs. 10 and 11), there was a marked reduction in the density of VIP binding sites in both the luminal and basal portions of the mucosa. This is in contrast to the binding sites located over the myenteric plexus and circular and longitudinal muscle which showed no significant changes compared to the histologically normal tissue. It should be stressed that the mucosa was present in all the ulcerative colitis tissues (e.g., Fig. 10g), although the density of VIP binding sites was greatly reduced.

DISCUSSION

Effector Role of Sensory Neuropeptides

The most important observation emerging from the present study is that expression of receptor binding sites for *specific* sensory neuropeptides is susceptible to functional perturbation in inflamed human tissue. Whereas sensory neuropeptides have been shown to play a role in conveying nociceptive information from peripheral tissues to the spinal cord, recent studies have suggested that sensory neurons also have an efferent function. Initially, it was noted that antidromic stimulation of peripheral sensory nerves caused a marked plasma extravasation dependent on intact sensory nerves (30,36). With the discovery that several neuropeptides were synthesized by sensory neurons (28), the question arose whether these putative neurotransmitters might be responsible for the neurogenic inflammation. A key observation, with regard to the action of sensory neuropeptides in peripheral tissues, was that sensory neurpeptides are transported not only to the terminals ending in the spinal cord but that the majority of the neuropeptide is transported to the peripheral terminal rather than the central end (7). These data, in conjunction with immunochemical data demonstrating that capsaicin [a neurotoxin relatively specific for the small, thinly myelinated or unmyelinated sensory neurons (63)] treatment could eliminate the sensory innervation of vascular beds in parallel with the disappearance of neurogenic inflammation (19,25), suggested that neuropeptide-containing sensory neurons (and in particular those containing substance P) have the proper anatomical relationship and physiological action on blood vessels to be agents involved in vasodilatation and plasma extravasation in peripheral vascular beds (32, 39, 40).

Relevance to Inflammatory Diseases

The possible relevance of the effector role of sensory neuropeptides to inflammatory diseases of the gastrointestinal (GI) tract (as diagrammed in Fig. 12) was suggested by capsaicin experiments demonstrating that blood vessels throughout the wall of the gastrointestinal tract are densely innervated by neuropeptide-containing sensory neurons (16, 17, 71, 78). These observations, and the finding in the canine GI tract that a substantial portion of submucosal arterioles, venules, and lymph nodules express receptor binding sites for SP (54,68), suggested that sensory neuropeptides regulate vasodilatation and plasma extravasation in the gastrointestinal tract and that dysfunction of this system might lead to an exaggerated inflammatory and immune response.

To test the relevance of this hypothesis to human inflammatory disease, we explored whether receptor binding sites for sensory neuropeptides are altered in two such diseases. While there are considerably more data suggesting that sensory neuropeptides are involved in two other common inflammatory diseases, i.e., arthritis (34,35) and asthma (39,41), we chose to explore changes in inflammatory bowel disease since this is one of the few human inflammatory diseases where surgical removal of the inflamed tissue is used to ameliorate the disease in severe cases. The use of surgical specimens was critical for the success of the present studies since it allowed us to perform receptor-binding experiments on human tissues in which degradation problems associated with long post-mortem time delays are not present (47).

Inflammatory bowel disease (IBD) is a generic term that refers to chronic inflammatory diseases of the intestine of unknown etiology, principally ulcerative colitis and Crohn's disease (70). Both these diseases share a number of clinical, epidemiologic, immunologic, and genetic features including: the most frequent age of onset is 15 to 30 years; there is a higher incidence in females than in males; there is an increasing incidence over the past 20 years; the diseases are more common in whites than nonwhites; they are most prevalent among Ashkenazi but not Sephardic Jews (61); and significant emotional events appear to be temporally related to the onset or exacerbation of these diseases (1). Since sensory neurons innervate the GI tract (16,78), are involved in regulating the inflammatory response in other disease states (14, 34, 38, 41), and there are other examples where sensory neurons alter their expression in response to stress [e.g., the expression of herpes simplex virus (10)], we investigated whether the location and levels of receptor binding sites for sensory neurotransmitters are significantly altered in IBD.

Of the nine receptor binding sites for sensory neurotransmitters examined, only SP and VIP showed significant changes, and the only cell types which demonstrated a dramatic increase in the expression of SP binding sites were those cells involved in regulating the inflammatory and immune responses. These data correlate well with previous findings on the action of SP. For example, SP-containing sensory terminals innervate both arterioles and venules. Upon release SP interacts with the receptor binding sites expressed by the smooth muscle and endothelium of arterioles and venules to produce vasodilatation and plasma extravasation, respectively (54). Thus, SP release in the muscle wall of arterioles would cause vasodilatation and an increase in pressure at the capillary bed, resulting in plasma extravasation. SP release in the vicinity of arterioles has been shown to cause dilatation (4), to produce changes in the permeability of the

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FIG. 6. A series of light and darkfield photomicrographs showing the localization of receptor binding sites for substance P (SP) in transverse sections of the human colon from normal (a, b, c) Crohn's disease (d, e, f) and ulcerative colitis (g, h, i) patients. Note that in the normal tissue a moderate concentration of SP binding sites is present over the circular muscle; this density does not change significantly in tissue obtained from the Crohn's disease and ulcerative colitis patients. In contrast to SP receptor binding sites present over blood vessels (small arterioles and venules, indicated by arrows), the germinal centers of lymph nodules (indicated by arrow heads) are virtually undetectable in the histologically normal tissue but reach the highest density of SP receptor binding sites in the entire human GI tract in the tissues obtained from the Crohn's disease and ulcerative colitis patients. Note also that blood vessels throughout the entire bowel wall display high concentrations of SP receptor binding sites in the Crohn's disease and ulcerative colitis patients. See Fig. 1 for further explanation. Line bar = 0.7 mm.


H-E
Normal
SubMc
CM
LM
a
H-E
Crohn's
LM
CM
SubMc
d
H-E
U.C.
SubMc
LM
CM
g
125I-SP
SubMc
CM
LM
b
125I-SP
LM
CM
SubMc
e
125I-SP
LM
SubMc
CM
h
Control
c
Control
f
Control
i

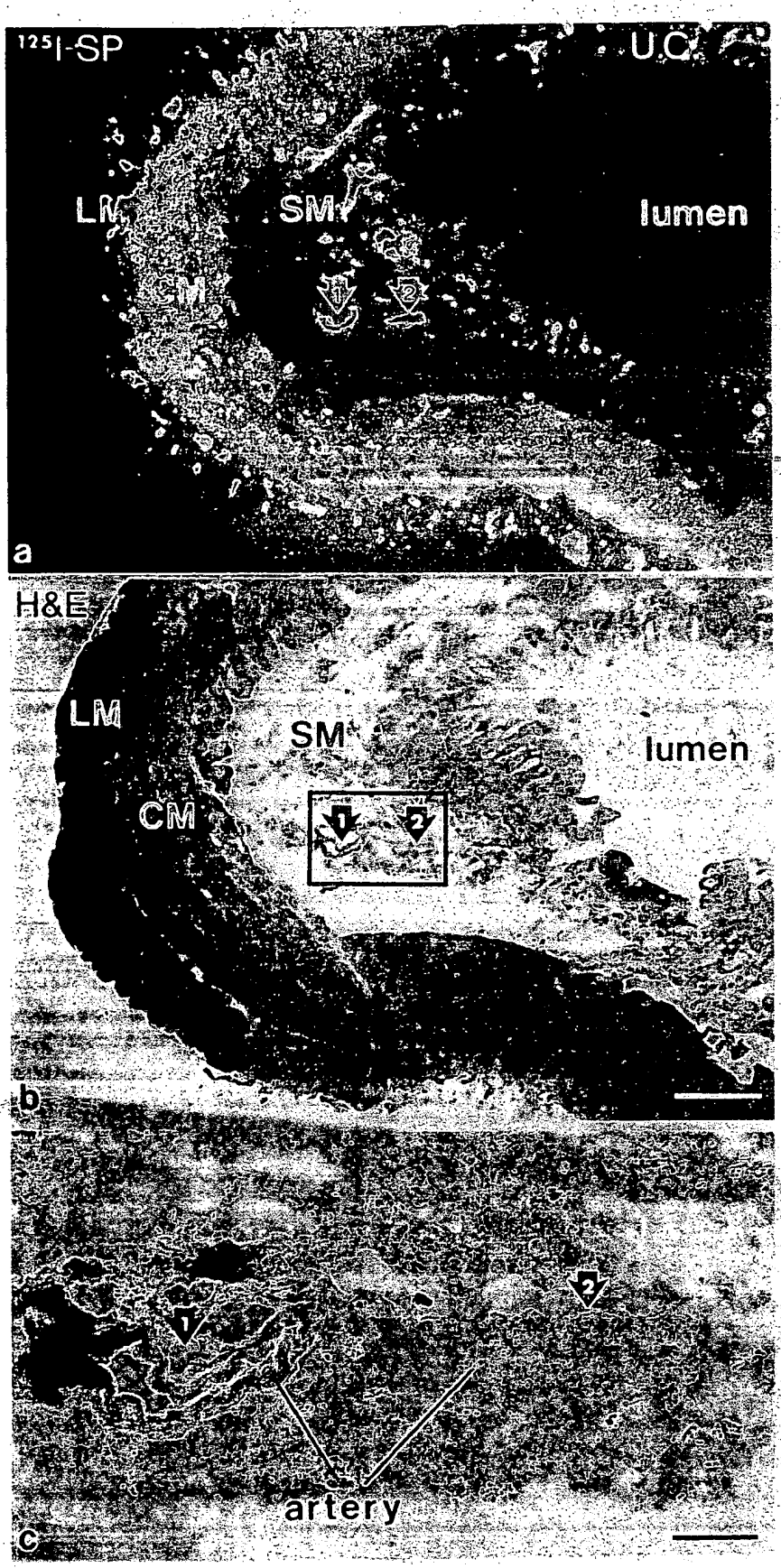
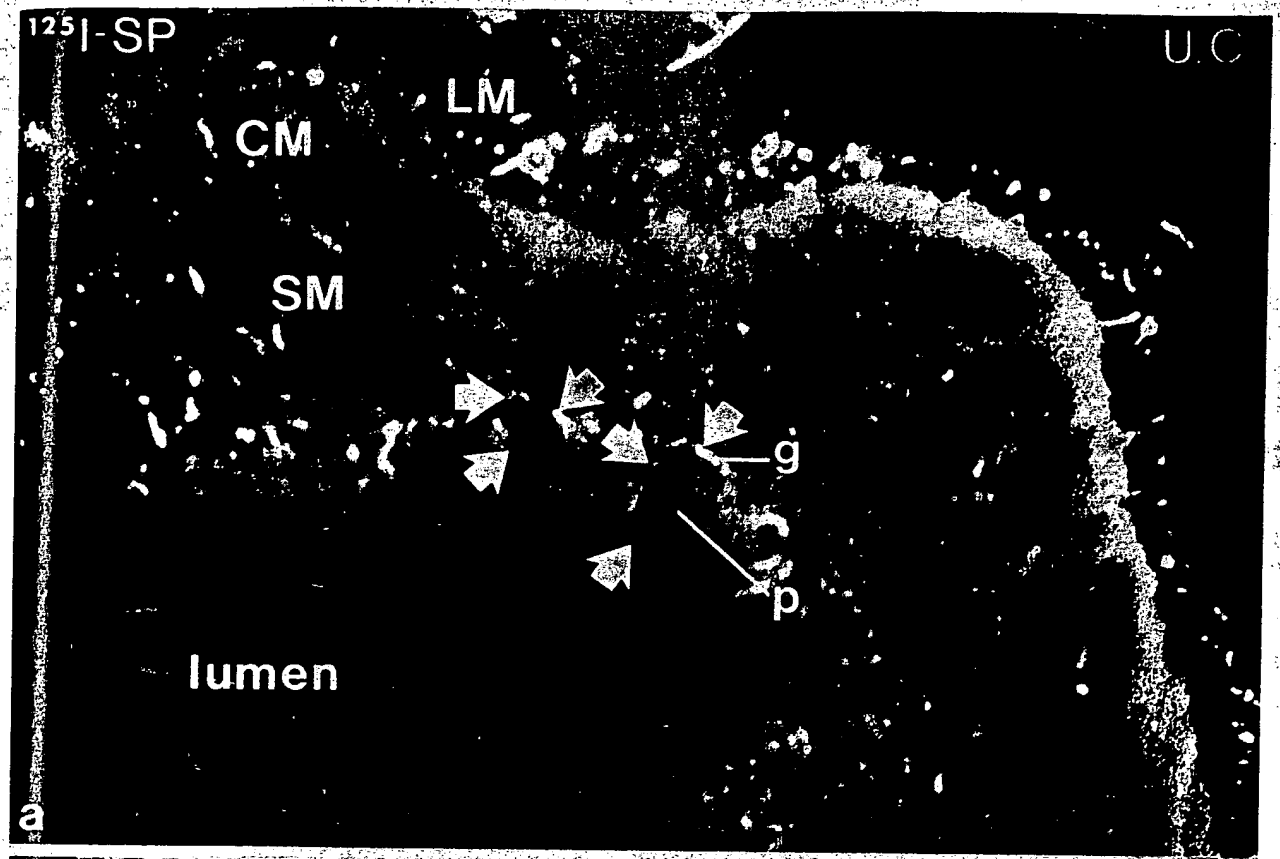
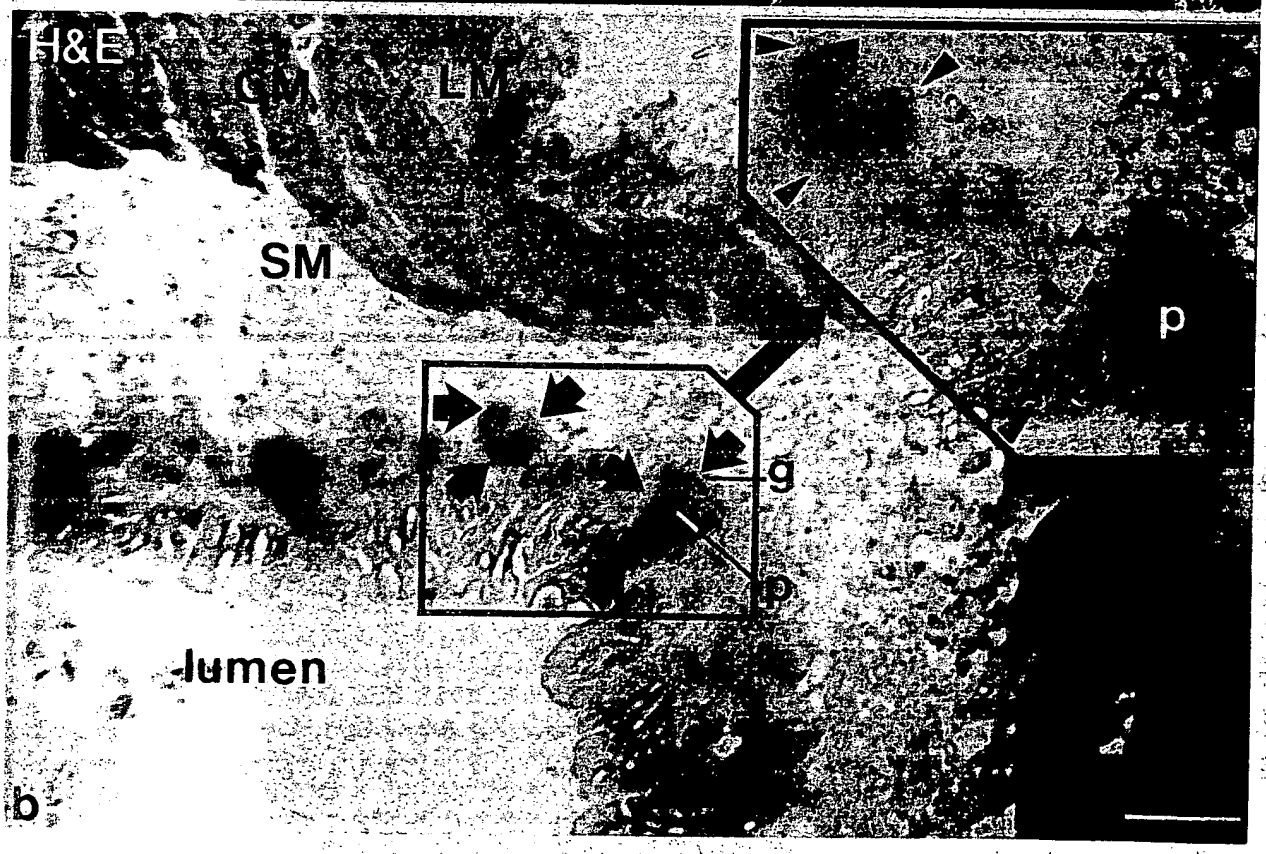


FIG. 7. A series of photomicrographs showing the detailed localization of specific SP binding sites on arteries in an inflamed colon of an ulcerative colitis patient. a. Darkfield autoradiogram showing the localization of SP binding sites on two arteries (arrows). b. Lightfield photomicrograph of H&E stained section of (a). Note that the two arteries in (b) correspond to the silver grains under the arrows in (a). c. High power enlargement of the inset in (b) showing the two arteries which express the SP binding sites. Line bar = 0.95 mm (b); 0.21 (c).


^{125}I-SP
U.C
LM
CM
SM
g
p
lumen
a


H&E
CM
LM
SM
p
g
p
lumen
b

PREVIOUS PAGE

FIG. 8. A series of photomicrographs showing the detailed localization of SP binding sites over the germinal center (g) but not the proliferative zone (p) of lymph nodules (outlined by arrows) in an inflamed colon of an ulcerative colitis patient. a. Darkfield autoradiogram showing the localization of SP binding sites over the lymph nodules. b. Lightfield photomicrograph of the H&E stained section of (a). Inset: High power enlargement of the area outlined in (b). Note that the only part of the lymph nodule that expresses SP binding sites in (a) is the lightly stained germinal center outlined by arrowheads in the inset in (b). Line bar = 0.9 mm (b).

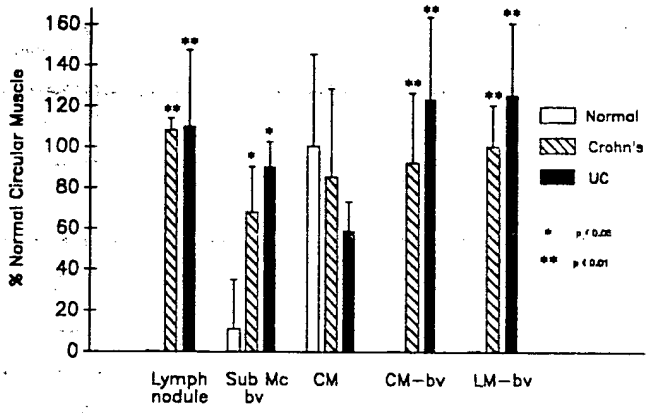


FIG. 9. Histogram showing the changes in the location and concentration of SP binding sites in the surgical specimens of normal colon obtained from carcinoma resection (open bar), Crohn's disease (hatched bar), and ulcerative colitis (filled bar). In this histogram 100% specific binding is that concentration of specific SP binding sites observed in the smooth muscle of the normal external circular muscle. Note that whereas in the histologically normal tissue SP receptor binding sites are undetectable in the blood vessels in the circular and longitudinal muscle and in the lymph nodules, in tissue from the Crohn's and ulcerative colitis patients these blood vessels and lymph nodules have the highest density of SP receptor binding sites in the entire human GI tract. See Fig. 2 for further explanation.

venules (30,39), and to alter the endothelium of the venules so that the circulating leukocytes attach to these vessels more readily [i.e., pavementing of leukocytes (21, 42, 72)]. These changes alter arteriolar smooth muscle tone and changes in the permeability and attachment properites of venules, which contribute to an influx of circulating inflammatory and immune cells into the inflamed area. Among the mobile elements known to invade the inflamed area are macrophages, mast cells, neutrophils, T-lymphocytes, and monocytes; all of these cells have been shown to express binding sites for SP and/or produce a functional response in the presence of SP (2, 21, 58, 67, 72, 76). These data further suggest that, in addition to promoting edema and the influx of leukocytes, SP may also have a direct action on the function of circulating leukocytes and thus directly regulate the immune response.

Equally suggestive of the contribution of SP to the underlying pathology in the disease is the apparent similarity in the location and concentration of SP binding sites between specimens obtained from patients on corticosteroid treatment vs. those from patients in which the treatment had been stopped for at least 6 months (Table 1). Since corticosteroids are often effective in treating the symptoms of IBD but not the underlying pathophysiology, these data further suggest that the ectopic expression of SP binding sites may be an underlying pathology contributing to the inflammatory state rather than simply being a result of the inflammation response.

Specificity of Receptor-Ligand Expression

Aside from the importance of these neuropeptide binding sites in regulating the inflammatory and immune responses, the present study also suggests that the expression of the appropriate binding site by the target tissue determines, at least in part, whether the released sensory neurotransmitter will produce a functional response. A population of sensory neurons which synthesize SP also synthesize and presumably corelease CGRP-α and CGRPβ (28, 40, 62), SK (23,44), CCK (12), and glutamate (13). It is believed that all branches of a neuron contain all the neurotransmitters synthesized by that neuron, regardless of whether or not a postsynaptic receptor is present (11,15). In other words, since many neurons are known to contain multiple neurotransmitters, it appears that the functional action of a given neurotransmitter in a particular branch of the neuron is ultimately dependent upon the presence of the appropriate postsynaptic receptor. Thus, in assessing what neurotransmitters regulate a particular peripheral organ or cell type, it is of paramount importance to first demonstrate that the target tissue expresses a pharmacologically relevant receptor binding site. An example of this is blood vessels in the normal guinea pig gastrointestinal tract, which although extensively innervated by SP-containing sensory nerves (16, 17, 78), do not contain detectable levels of SP receptors (8, 48), and show no increase in vascular permeability to either direct application of SP or to antidromic activation of the sensory nerves innervating these tissues (39). In other words, the permeability effects of SP are determined by the presence of SP receptors on blood vessels, and this expression of SP receptors (at least on gastrointestinal blood vessels of man) preferentially occurs in an inflammatory state. These data also suggest that receptor binding sites for other sensory neurotransmitters may play a role in regulating human peripheral tissues in both normal and diseased states, but this role will only be evident when examined in the proper physiological or pathological context.

Receptor-Ligand Mismatch

These results also have clear implications for the receptor-

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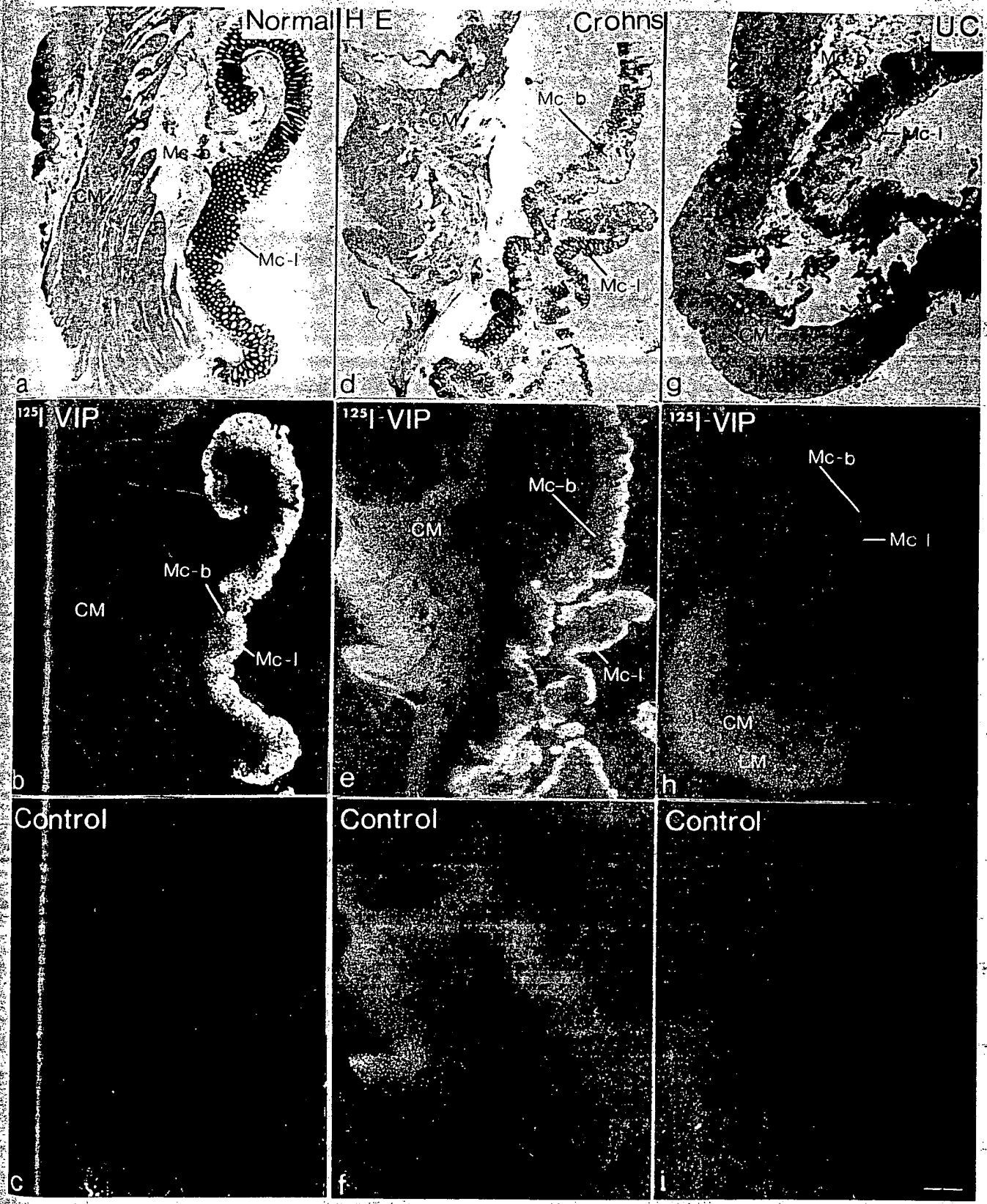
FIG. 10. A series of light and darkfield photomicrographs showing the localization of receptor binding sites for vasoactive intestinal polypeptide (VIP) in the human colon for normal (a, b, c), Crohn's disease (d, e, f), and ulcerative colitis (g, h, i) patients. The highest concentration of specific binding sites is present over the luminal aspect of the mucosa (mc-l) whereas a low concentration of receptor binding sites is present over the basal aspect of the mucosa (mc-b) and the external circular (CM) and longitudinal (LM) muscle. Note that the one highly significant difference in the autoradiograms is that the luminal mucosa of the ulcerative colitis tissue contains very low levels of VIP binding sites compared to either the normal or Crohn's disease tissue. See Fig. 1 for explanation. Line bar = 0.7 mm.


Normal
H E
Crohns
U.C.
Mc-b
CM
Mc-l
a
d
g
125I-VIP
125I-VIP
125I-VIP
Mc-b
CM
Mc-l
b
e
h
Control
Control
Control
c
f
i

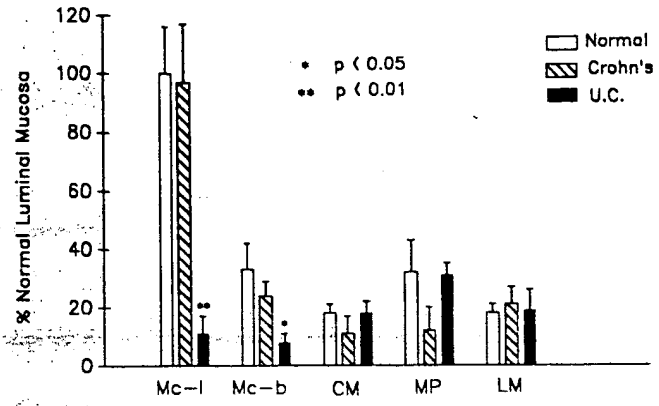


FIG. 11. Histogram showing the changes in the location and density of specific binding sites for vasoactive intestinal polypeptide (VIP). Specific VIP binding sites are over the luminal aspect of the mucosa (mc-l) whereas a low concentration of receptor binding sites is over the basal aspect of the mucosa (Mc-b) and the external circular muscle (CM), myenteric plexus (MP), and external longitudinal muscle (LM). Note that the mucosa (both the luminal and basal aspect) of the ulcerative colitis tissue contains a very low concentration of VIP binding sites compared to either the normal or Crohn's tissue. See Fig. 1 for further explanation.

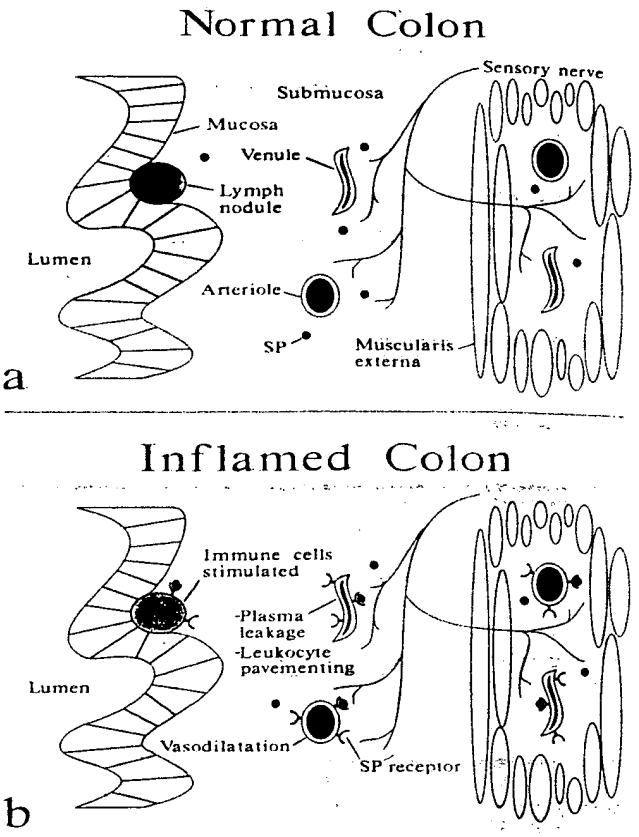


FIG. 12. Schematic diagram of the major differences observed in the present study between (a) the normal human colon and (b) the inflamed colon in inflammatory bowel disease (IBD). The most notable difference is the ectopic expression of high concentrations of SP receptor binding sites by arterioles, venules, and lymph nodules in IBD vs. the normal colon. We hypothesize that this ectopic expression of SP receptor binding sites is involved in regulating the vasodilatation of the arterioles, plasma extravasation and influx of leukocytes through the venules, and a hyperimmune response from direct SP stimulation of leukocytes, all of which are common to these and other human inflammatory diseases.

ligand mismatch problem. The presence of a nerve fiber containing a particular transmitter is often not well correlated with the presence of an appropriate postsynaptic receptor (22, 50, 51, 57). SP-immunoreactive fibers innervate arterioles and venules throughout the normal human colon, and the distribution of immunoreactive SP fibers and levels of SP change only minimally [2-fold increase (29)] or not at all (6, 66, 74) in the inflamed colon in patients with ulcerative colitis or Crohn's disease. This is in contrast with dramatically elevated (1000–2000-fold) levels of SP receptor binding sites expressed by arterioles, venules, and lymph nodules in inflamed colon. Since it appears that SP binding sites in blood vessels and lymph nodules in the human colon reach detectable levels only in the inflamed state, it is difficult to assess the full repertoire of the target tissues of a particular neurotransmitter unless one knows a priori in what context the expression of binding sites should be examined. It is also conceivable that a receptor is tonically present and only in a disease state is its ligand expressed or released by the presynaptic nerve terminal. Thus, when a mismatch is observed between the presence of a receptor and the presence of its presumed ligand, it should be considered that a proper match may only be apparent in a different physiological or pathological context. The present data also suggest that the coordinated synaptic localization of the native ligand and its receptor must involve factors other than just the presence of the ligand since in the normal colon the innervation of blood vessels by SP apparently does not by itself induce the expression of SP receptors.

Substance P as a Multifunctional Effector Peptide

In the present report we have demonstrated the ectopic expression of high densities of SP binding sites by arterioles, venules, and lymph nodules and suggest that, in a pathological state, SP and its receptors play a role in regulating the inflammatory and immune responses characteristic of inflammatory diseases. A key question that remains to be answered is what is the normal function of the neuropeptide-containing sensory neurons? One observation which suggests a normal function for these neuropeptide-containing sensory neurons is that one of the most pronounced deficits in neonatal capsaicin-treated rats (the neurotoxin destroys primarily the neuropeptide-containing C-fibers) is that the skin and fur lose their luster and that these animals *lack* a normal wound healing response; i.e., these animals have numerous small wounds which do not appear to be healing. Such a lack of the normal trophic and/or wound healing response after sensory denervation can also be inferred from the ulceration of the cornea which follows trigeminal deafferentation (5). Together the observations suggest that, in a pathological state, SP appears to be involved in regulating a hyperinflammatory and immune response and that, in the normal condition, these sensory neuropeptide-containing neurons may have a trophic action on tissues they innervate and also regulate wound healing after injury.

As reviewed above, SP and other sensory neurotransmitters appear to be released centrally in the spinal cord to signal pain and peripherally to produce vasodilatation, plasma extravasation, and homing of leukocytes to the area of injury. Since there cannot be tissue repair until there has been an appropriate inflammatory and immune response (18), the initial action of SP may be to promote and direct the inflammatory and immune responses in the damaged tissue. After the infection and damaged tissue have been cleared.

SP and other neuropeptide growth factors continue to be released, but their effects would now be directed towards mitogenesis and tissue remodeling. Two sensory neuropeptides, SP and neurokinin A, have been shown to be potent mitogens for fibroblasts in culture (64). The key to this model is that SP should be both stimulatory and inhibitory towards the same cells, depending on the context of other chemical signals present. This multifunctional role for peptide growth factors has clearly been shown for other peptides, including transforming growth factor-β, which stimulates growth of certain fibroblasts in vitro in the presence of platelet derived growth factor, but inhibits the growth of the same cells if epidermal growth factor is present (69,75). Therefore, in human inflammatory diseases it may be that ectopic expression of SP or its receptor is not the primary pathology, but rather that the pathology may involve whatever other factors are needed to switch SP action from the "catabolic" mode, where inflammatory and immune responses are promoted, to the "anabolic" process of tissue growth.

Conclusions

There is an elegance in the simplicity of assigning the same neuron and perhaps the same neuropeptide for roles in nociceptive information transmission and simultaneous peripheral control of recovery from injury. The normal function of the small thinly myelinated sensory neuron may be to signal tissue damage to the brain and to regulate normal cell turnover and, in the case of injury, the subsequent inflammatory, immune, and would healing response in the damaged tissue. Such an organization would allow a coordination between the biochemical processes of wound healing and the behavioral response to injury so that use and further injury of the damaged tissues does not occur during the repair process.

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